(54) Title: METHOD FOR PRODUCING FACTOR VIII:c-TYPE PROTEINS

(57) Abstract

An improved method for producing Factor VIII:c-type proteins which involves culturing mammalian cells which are capable of expressing the protein. In accordance with this invention the cells are cultured in a medium containing an effective amount of a substance comprising (a) von Willebrand Factor (VWF), (b) a phospholipid or phospholipid mixture, or a mixture of (a) and (b).

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METHOD FOR PRODUCING
FACTOR VIII:c-TYPE PROTEINS

Background of the Invention

The Factor VIII complex has two distinct biologic
functions: coagulant activity and a role in primary hemo-
statis. The analysis of Factor VIII deficiency diseases,
classic hemophilia and von Willebrand's disease, have
contributed to the understanding that Factor VIII is a
complex of two components. The Factor VIII:c procoagulant
protein (antihemophilic factor) and the Factor VIII related
antigen (von Willebrand factor,VWF) are under separate
genetic control, have distinct biochemical and immunologic
properties, and have unique and essential physiologic
functions.

The Factor VIII:c molecule is an important regulatory
protein in the blood coagulation cascade. After activation
by thrombin, it accelerates the rate of Factor X activation
by Factor IXa, eventually leading to the formation of the
fibrin clot. Deficiency of Factor VIII:c (classic hemophilia)
is an X-linked chromosomal disorder that has been a major
source of hemorrhagic morbidity and mortality in affected
males. Treatment usually consists of frequent transfusions
with blood products. The latter has resulted in a high
incidence of infectious complications (such as various
forms of hepatitis and acquired immunodeficiency disease)
in the hemophiliac population.

The VWF molecule is an adhesive glycoprotein that
plays a central role in platelet agglutination. It serves
as a carrier for Factor VIII:c in plasma and facilitates
platelet-vessel wall interactions. Discrete domains of VWF
which bind to platelet receptor sites on glycoprotein 1b

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and on the glycoprotein IIb-IIIa complex, as well as binding sites on collagen have been noted. VWF is made up of multiple, probably identical, subunits each of 230,000 daltons. VWF is synthesized in endothelial cells and megakaryocytes. In the plasma it exists as high molecular weight multimers ranging from 5×10^5 to 10^7 daltons. The Von Willebrand factor contains 5-6% complex carbohydrate, which appears important in the molecules ability to bind platelets. A variety of abnormalities in VWF activity can result in Von Willebrand's disease. The disorder is generally inherited in autosomal dominant fashion and may affect as many as one in 2000 individuals. Mild forms of the disease frequently go undiagnosed, whereas severely affected patients may require frequent blood product support with its associated risks.

Recently, the isolation of the genes for both factor VIII:c and Von Willebrand factor have made possible the production of recombinant factor VIII:c and VWF preparations which are essentially free of contaminating viruses (Toole et al. 1984, Wood et al. 1984, Lynch et al. Cell 41:49-56 1985, Ginsberg et al. Science 228:1401-1406 1985). The production of Factor VIII:c and analogs thereof through recombinant DNA technology has been achieved utilizing mammalian cells as a recipient of DNA encoding Factor VIII:c (or analogs thereof) contained in appropriate expression vectors. Primary concerns for the synthesis of a recombinant Factor VIII:c include (i) the yield of recombinant FVIII:c obtainable from the culture medium, (ii) the stability of the recombinant FVIII:c so produced, (iii) the efficiency and cost of purification of the FVIII:c and (iv) the overall cost of producing purified recombinant FVIII:c.

For best results, we have typically cultured FVIII:c-producing cells in media containing mammalian serum, e.g. conventional preparations of fetal bovine serum, in amounts

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of about 10% serum by volume relative to total media volume.
(For the sake of simplicity, all serum concentrations
hereinafter are expressed as a volume % of total media.)
We have found that in the absence of such serum supplements
both the yield and stability of the recombinant FVIII:c suffer
significantly. However, the cost of serum supplements and
the added inconvenience and expense in purification resulting
from the use of serum rendered the use of serum an
undesireable necessity and the wide scale use of recombinant
FVIII:c a commercially less attractive alternative to
natural FVIII:c purified from plasma.

    After extensive experimental modifications of media
for FVIII:c-producing cells we have surprisingly found a
method for producing higher yields of stable FVIII:c-type
proteins (as described hereinafter) using media containing
reduced amounts of serum (e.g.,semi-defined media) or
essentially serum-free media (defined media). We have
found that host cells producing FVIII:c-type proteins
produce recoverable, stable FVIII:c-type proteins in semi-
defined and defined media in yields at least comparable to
those obtained in the presence of 10% serum if the semi-
defined or defined media contains a suitable amount of a
hydrophobic substance such as VWF or certain phopholipids.
We have further found that FVIII:c-type proteins produced
in semi-defined or defined media lacking such supplements
typically exhibit dramatic instability and are recoverable
in extremely low yield if at all.

    Evidence to date suggests either that VWF may have a
stabilizing effect _in_ _vivo_ on the Factor VIII:c in plasma,
or that the VWF can ellicit the _in_ _vivo_ release from storage
depots or stimulate the _in_ _vivo_ synthesis and/or secretion
of Factor VIII:c (Weiss, H. J. et al., 1977,J. Clin. Invest.
_60_: 390-404). It has also been suggested that thrombin-
activated Factor VIII (derived from natural, human FVIII)
is stabilized by phospholipids, presumably with respect to

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thrombin-mediated degradation. See Andersson and Brown, 1981, Biochem. J. 200:161-167. However, to our knowledge there is no suggestion in the prior art as to any possible effect(s) of VWF or phospholipid on the in vitro production of Factor VIII:c-type proteins (where, for example, thrombin is substantially absent) or any suggestion of media supplements comprising VWF and/or phospholipids substantially free from the complex mixture of components present in mammalian serum in accordance with this invention.

Summary of the Invention

This invention concerns an improved method for the production of Factor VIII:c-type proteins.

"Factor VIII:c-type" proteins, as the term is used herein, means proteins exhibiting Factor VIII:c-type procoagulant activity. Factor VIII:c-type proteins within the ambit of this invention are encoded for by DNA sequences capable of hybridizing to DNA encoding Factor VIII:c. In addition to natural mammalian, e.g. human, Factor VIII:c, Factor VIII:c-type proteins include, for example, proteins which contain deletions of one or more amino acids between the 90 Kd and 69 Kd cleavage sites with respect to native Factor VIII:c, as described in greater detail in International Application No. PCT/US86/00774, published 23 October 1986. Factor VIII:c-type proteins also include Factor VIII:c analogs containing amino acid deletions between the 50/40 cleavage site and the 69 Kd cleavage site which may be produced by methods analogous to those disclosed in PCT/US86/00774. Factor VIII:c-type proteins further include analogs (with or without deletions as mentioned above) wherein one or more of the cleavage sites spanning arginine residues at positions 226, 336, 562, 740, 776, 1313, 1648 or 1721 have been rendered resistant to proteolytic cleavage, e.g., by replacement of one or

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more amino acids with different amino acids by conventional site-directed mutagenesis of the cDNA to be expressed. Factor VIII:c-type proteins thus include natural Factor VIII:c, "recombinant" Factor VIII:c and analogs thereof having procoagulant activity, and non-recombinant Factor VIII:c or analogs thereof produced by cell lines derived from cells which produce the protein.

The method of this invention thus utilizes mammalian cells which contain DNA encoding a Factor VIII:c-type protein and which are capable of expressing the protein. In accordance with the method of this invention the cells are cultured in media containing an effective amount of a stabilizing substance for a Factor VIII:c-type protein. Such substances include: (i) mammalian von Willebrand Factor (VWF), (ii) a stabilizing phospholipid or phospholipid mixture, and (iii) mixtures of VWF and phospholipid(s). Preferred effective amounts of VWF range from about 0.1-10 ug VWF/ml media, with 1-3 ug/ml being specially preferred. One readily obtainable source of suitable phospholipids comprises commercially available dry milk preparations such as dried skim milk and low-fat skim milk. Such dried milk preparations may be added to the media in amounts ranging from about 0.01% - 10% (weight of dry milk/volume of media). For optimal effect on Factor VIII production with minimal toxic effect on the cells, about 1% - 3% dry milk is presently preferred. The dry milk preparations may be conveniently sterilized by first preparing a 10% aqueous suspension of the milk and autoclaving. Another readily obtainable source of suitable phospholipids is commercially available soybean lecithin, which may be added to the medium in accordance with this invention preferably in liposome form.

"Phospholipid" as the term is used herein means an ester of phosphoric acid containing one or two molecules of fatty acid, an alcohol and a nitrogenous base. Examples of such phospholipids include Cephalin, phosphatidyl serine:

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phosphatidyl choline mixtures, phosphatidyl inositol, phosphatidyl ethanolamine, soybean lecithin and mixtures thereof, with soybean lecithin being especially preferred. Other phospholipids useful in this method as well as effective and/or optimal concentrations and/or mixtures thereof may be readily identified by those skilled in the art using methods described in greater detail hereinafter. Presently preferred effective amounts of phospholipid or phospholipid mixtures comprise about 1-1000 ug phospholipid or phospholipid mixture per ml of culture media, with concentrations greater than about 100 ug/ml being more preferred and concentrations between about 200-300 ug/ml being especially preferred. Additionally, it is presently preferred to add the phospholipid mixture to the culture media in the form of liposomes, preferably having a diameter of up to about 500 nm. Preferably the liposomes are unilamellar, although multilamellar liposomes may also be used. Most preferably the diameter of the liposomes is less than about 100 nm. Furthermore, liposomes made by conventional methods from said phospholipids may be used, either in admixture with or containing the Factor VIII:c-type protein, as a carrier or vehicle for administering the protein to patients. Where dried milk is used as the source of phospholipids, the dried milk may be added directly (rather than in the form of liposomes) to the media.

VWF may be obtained from mammalian, e.g., human, bovine, porcine, etc., serum by conventional methods or from cell lines derived from VWF-producing cells such as endothelial cells. Alternatively, "recombinant" VWF (i.e., VWF derived from genetically engineered cells) may be used. In one embodiment, VWF-producing cells, such as cells suitably engineered to produce VWF, are cultured in the medium to condition it with VWF either prior to or simultaneously with the culturing of cells which produce a Factor VIII:c-type protein. Alternatively the recombinant VWF may be

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separately produced and added as an exogenous supplement to the media to be used for culturing the cells producing the Factor VIII:c-type protein. In another embodiment the cells which produce Factor VIII:c-type protein are suitably engineered, i.e. effectively transformed with a VWF transcription unit, such that the VWF and the Factor VIII:c-type protein are co-expressed by the same cells. In a further embodiment of this invention the media used for culturing the cells producing the Factor VIII:c-type protein contains both VWF, by virtue of one of the above-mentioned processes, and stabilizing phospholipid(s). In that case, it may be desirable to use reduced amounts of each component relative to the amounts used if used alone. By using appropriately supplemented defined media in accordance with this invention high levels of recoverable, stable Factor VIII:c-type activity are produced which may then be recovered and purified without the necessity for separation of serum components therefrom. The culture media used in this invention may additionally contain mammalian-derived serum, e.g. fetal bovine serum, preferably in amounts less than about 10%, more peferably in amounts less than about 5%, and even more preferably in amounts between 0 and 1%, although essentially serum-free media is especially preferred. Other conventional mammalian cell culture media supplements may also be added.

This invention is illustrated in the following examples which set forth typical procedures demonstrating, among other things, the ability to overcome the serum dependence in the production of recoverable, active Factor VIII:c-type proteins using phospholipids and VWF as media supplements. The examples are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set froth in the claims which follow thereafter.

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EXAMPLE I

Establishment of Chinese Hamster Ovary Cell Lines which Express Human Factor VIII:c

The Factor VIII:c expression plasmid used in this Example was RxPy VIII-I which contains in clockwise order the polyomavirus enhancer, the first leader sequence of the adenovirus tripartite leader sequence, a Factor VIII:c transcription unit followed by a DHFR gene and SV40 poly-adenylation signal, and a gene encoding tetracycline resistance. This plasmid was introduced into dihydrofolate reductase (DHFR) deficient Chinese hamster ovary cells by cotransformation with a DHFR expression plasmid and subsequent selection for cells that grow in the absence of added nucleotides. One particular pool of transformants designated lig 1 was subsequently grown in increasing concentrations of methotrexate (MTX) in order to amplify the DHFR and Factor VIII genes. The resultant cell line expressed high levels of Factor VIII activity as determined by either the ability to clot Factor VIII deficient plasma [Clotech (APPT) assay] or by the ability to generate Factor Xa in the presence of Factor IXa, phospholipid, calcium, and Factor X (Kabi Cotest assay). The ability of these CHO cells to produce Factor VIII:c is shown in Table I. The Factor VIII activity increased 10,000 fold with increasing levels of MTX resistance which correlated with the Factor VIII gene copy number. Other expression vectors may also be used in place of RxPy VIII-I so long as they are capable of directing the expression of Factor VIII:c or analogs thereof. Such vectors include, for example, pCVSVL2-VIII (ATCC No. 39813, see European Application No. 85202121.1) and pDGR-2 (ATCC No. 53100, see PCT/US86/00774-deletion analog). Other Factor VIII:c expression vectors may be prepared using conventional expression vectors and techniques containing, for example, the SalI fragment from pCVSVL2-VIII or pSP64-VIII (ATCC No. 39812). The SalI fragment from either vector contains a DNA sequence encoding full-length Factor VIII:c.

Table I: Factor VIII Expression in Transfected and Amplified CHO cells

Pool	MTX (uM)	mU/ml/day of VIII:c
Lig 1	0.0	0.1
	0.02	11.5
	0.1	88.0
	1.0	288, 545*
	5.0	644, 875*
	20.0	1075

*Represents samples from two independent assays

Plasmids pAdD26SVpA(3) (Kaufman and Sharp, 1982, Mol. Cell. Biol.) and plasmid pRXPy-VIII-I were digested with Cla 1 and the resultant linearized DNA was ligated in vitro and coprecipitated with CaPO4 and used to transfect CHO DHFR deficient DUKX-BII cells. Cells which efficiently expressed DHFR would be expected to contain the enhancer element from pRXPyVIII-I associated with the DHFR gene from pAdD26SVpA(3). Results have been consistent with this hypothesis. Subsequent selection for increased DHFR expression by propagation of the cells in increasing concentrations of MTX results in cells which have amplified the Factor VIII gene and the DHFR gene. At each level of MTX selection, samples of the conditioned media (approximately 10^6 cells/ml in alpha media supplemented with 10% fetal bovine serum) were taken for Factor VIII:c activity assay determined by the Kabi Coatest method modified to obtain sensitivity better than 0.05 mU/ml. Comparable results were also obtained by the one-stage activated partial

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thromboplastin time (APTT) coagulation assay using Factor VIII:c deficient plasma. All samples exhibited thrombin activation of 30 - 50 fold. For thrombin activation the samples were pretreated 1 -10 min with 0.2 units/ml thrombin at room temperature.

EXAMPLE II:Serum Dependence of Factor VIII:c Synthesis:

CHO cells (Lig 1 2 A subclone B10 in .1 uM MTX at 80% confluence) which are rinsed and fed with media containing 10% FCS or defined media (serum free, containing: 5 mg/ml BSA, insulin, transferrin, selenium, hydrocortisone and putrescine) accumulate Factor VIII activity. The rate of appearance in defined media is roughly 4-fold lower than in serum-containing media. The 4-fold difference becomes larger as the cells are propagated in the absence of serum. This results from inefficient rinsing of the cells. The rate of Factor VIII:c appearance increases fairly linearly up to 24 hrs. which suggests the VIII is stable in the media. This result is similar to results obtained with COS cells at lower levels of VIII expression (Approx. 10mU/ml/day)

Cephalin, a mixture of phospholipids, can counteract at least part of the serum deficiency. CHO cells (Lig. 2 A pool in 20uM MTX) were separately cultured with and without serum and rinsed after 4 hr. The two CHO cultures were then split again, and a portion of the serum$^+$ and of the serum$^-$ CHO cells were supplemented with 5uM cephalin for an additional 2 hr. The other portion of the serum$^+$ and serum$^-$ CHO cells were not supplemented with cephalin. Media was assayed at 6 hrs. and 25 hrs. and results shown below:

Conditions		FVIII:c Activity*	
Serum	Cephalin	6 hr.	25 hr.
+FCS	+Ceph	594	1044
-FCS	+Ceph	408	514
+FCS	-Ceph	563	1492
-FCS	-Ceph	140	372

*mU/ml

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The results suggest that cephalin alone can increase VIII activity in the absence of serum but that its effect is short lived (i.e. observed after 2 hrs. but is diminished at 25 hrs.). In one experiment the concentration of cephalin was increased and there was no further increase in VIII activity. This indicated some component in the cephalin was not rate limiting.

Part of the cephalin effect can be elicited by a simpler mixture of phospholipids or by single phospholipids. Cells (Lig 1 2 A .02 pool in 20uM MTX) were fed with 10% fetal calf serum or serum free media for 24 hrs. and then either cephalin (5um) or a mixture (1:4) of phosphatidyl serine and phosphatidyl choline (PCPS) were added to serum free cultures. Results from media assayed after 2 hrs. were:

conditions	FVIII:c Activity
Serum Free	110
Serum Free + Cephalin	489
Serum Free + PCPS	230
10% FCS	613

This result demonstrates that phospholipids alone can increase VIII activity in conditioned media.

Analysis of the thrombin activability of VIII expressed in CHO cells growing under different conditions suggests that the presence of serum decreases the degree of thrombin activation. CHO cells (Lig 1 2 A pool in 20uM MTX) were rinsed and fed with media containing 10% fetal calf serum or defined media (5mg/ml BSA, transferrin, selenium, insulin, hydrocortisone, putrescine). 24 hrs. later either cephalin or 10% FCS was added and 2 hrs. later samples taken for assay and measure of thrombin activatibility:

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| Sample Media | Added at 24 h | Assay at 26 hrs. | | Coagulation Assay (fold activation) |
		Cobas mU/ml	mU/ml	
Defined media	-	315	300	20X
Defined media	5uM cephalin	752	1040	34X
Defined media	10% FCS	684	440	8.4X
+ 10% FCS	-	1078	1200	10X
+ 10% FCS	5uM cephalin	1154	1120	14X
Defined media	10% boiled FCS	543	-	-

These results indicate that the presence of serum
increases the activity produced compared to serum free media
but reduces the thrombin activatibility. In contrast,
cephalin can compensate for the serum effect on increasing
the activity of the VIII produced but does not reduce the
thrombin activatibility. Thus, in serum free media, with
the addition of cephalin 2 hrs. prior to harvest, CHO cells
produce VIII at 1 unit/ml and this material exhibits a 34
fold thrombin activation. This experiment also demonstrates
that 10% FCS added to serum free media 2 hrs. prior to
assay can also increase the amounts of VIII activity. This
ability was not dimimished by boiling the serum 10 min prior
to its addition. Thus, the serum factor required for VIII
activity is heat stable. We conclude that the serum factor
required for increasing VIII may comprise two components: a
phospholipid and another, heat stable factor which may be
required to stabilize the phospholipid.

To determine whether the 10% serum was limiting for
Factor VIII:c expression in the highly amplified CHO cell
lines, we monitored the effect of increasing amounts of
serum on the ability to elicit factor VIII:c activity in
the cell line 10A1. 10A1 is a clone derived from selection
of the Lig 1 pool for growth in 1 mM MTX. This experiment

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demonstrated the effect on Factor VIII activity of adding increasing amounts of fetal bovine serum to the 10A1 cells for 24 hrs. 50% serum yielded three-fold more activity in the 24 hr. conditioned media compared to 10% serum. Other results have indicated that the amount of active Factor VIII antigen is correspondingly increased when cells are propagated in 50% serum. Other cell lines, which express slightly lower levels of Factor VIII:c show less dramatic increases in Factor VIII:c activity upon growth in higher concentrations of serum. Thus there appears to be some limiting requirement for Factor VIII: expression in higher producing cells such as those which would be desirable for commercial-scale production of Factor VIII.

Example III
Serum Dependence of rFactor VIII:c Synthesis in Suspension Cultures of CHO.

The following table illustrates the dependency of recombinant Factor VIII:c (rFVIII) activity on serum levels in culture. A relatively low rFVIII producer, clone 1E6, was grown in suspension culture for 3 to 4 days in medium containing various concentrations of fetal bovine serum (FBS).

Serum Concentration in Medium*	rVIII Activity (mU/ml) after 3-4 days	Average Productivity (U/10^6 cells/day)
10% FBS	318	0.19
5% FBS	100	0.03
Defined*	4	1/4-0.01

* RPMI 1640 was employed as basal medium for all of the above. The defined medium consists of insulin, 5ug/l; transferrin, 5ug/ml; serenium, 5ng/ml; hydrocortisone, 10^{-8}M, putrescene, 100ng/ml; BSA, 5mg/ml.

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The same serum dependence has been observed with other rFVIII secreting CHO cell lines. These results do not reflect genetic instability since original expression levels can be regained on addition of serum.

We have found that addition of phospholipid to culture medium can replace the serum requirement, however relatively high concentrations of phospholipid are required (on the order of 10-20 fold higher than previously used with serum-containing media). With respect to the dependence of the recovery of FVIII activity on phospholipid concentration, we have found that supplementing defined media (DM) with 240 ug soybean lecithin (SL)/ml media yielded (after 24 hrs) about twice as much FVIII activity as was obtained in DM containing 160 ug SL/ml and five times as much FVIII activity as was obtained in DM containing 80 ug SL/ml. Nonetheless, DM containing 80 ug SL/ml provided measurably more FVIII activity than DM containing 1% Fetal Bovine Serum(FBS)(semi-defined media), while the semi-defined media provided significantly more FVIII activity than did DM alone. Significantly, the level of rFVIII generated over a 24h period in defined medium in the presence of 240 ug SL/ml media is at least as high as that generated in DM supplemented with 10% FBS. Increasing the concentration of SL above 240ug/ml resulted in no further increase in rVIII activity in this experiment. Illustrative results of one experiment are shown below:

Media	rFVIII activity after 24h
Defined Media (DM)	~ 40 mU/ml
DM + Soybean lecithin (SL) (240 ug SL/ml media)	~ 270 mU/ml
Media containing Fetal bovine serum (FBS) (10% FBS)	~ 200 mU/ml

This data illustrates the increase in the rFVIII:c activity obtained with relatively high concentrations (e.g., 240 ug/ml) of soybean lecithin phospholipid in the absence of fetal bovine serum. CHO cells (1E6 in 0.1 umolar MTX) were suspended at a concentration of 3 x 10^5 cells/ml in defined medium containing 5g/l of bovine serum albumin either in the absence or presence of phospholipid or medium containing 10% FBS for 24h at 37 C. At the conclusion of the incubation samples of cell-free conditioned medium were assayed for rFVIII:c activity by a chromogenic assay.

We have found that the addition of phospholipid in amounts up to about 320 ug/ml media causes no marked changed in cell growth in either defined or semi-defined media. In one set of experiments we found that maximum rFVIII activity was obtained in the culture where 320 ug phospholipid/mL media was added. In semi-defined medium, maximum levels were obtained after 72h where 240 ug/mL of soybean lecithin was added. These and other data illustrate that soybean lecithin added stepwise to cultures on days 0, 1, 2 and 3 allowed production of rFVIII. The optimum concentrations were around 240 ug/mL in these and other experiments regardless of the method of preparation of the phospholipid.

The cellular productivities in two different CHO cell lines from experiments similar to the above are shown below.

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Medium	Average Productivity (u/10^6 cells/day)	(Cell Line - 1E6)
10% FBS	0.19	
5% FBS	0.03	
Defined	1/4-0.01	
Defined + SL	0.24	
Defined + 1% FBS + SL	0.25	

Medium	Average Productivity*	(Cell Line - H9.05)
10% FBS	0.43	
Defined + 1 % FBS + SL	0.51	

*Units as above

Thus, productivities of rFVIII from rCHO cells are at least equivalent in defined medium supplemented with phospholipid as in serum containing medium. However, as illustrated by the data above, the bulk quantity of rFVIII produced in defined medium is less than in serum containing medium. This is due to the fact that cells grow more rapidly and to higher cell densities in serum-containing medium rather than being more productive. On supplementation of defined medium with small quantities of serum (e.g. 1%) cell growth is improved. Indeed, after a short period of adaption cells will grow almost as well in semi-defined medium as in 10% FBS supplemented medium. We have found that rCHO cell lines (e.g., our H9.05) grow to similar cell densities and are at least equally productive (in Factor VIII) in phospholipid supplemented semi-defined medium as in 10% FBS supplemented medium.

Furthermore, we have also examined the physical

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nature of the soybean lecithin preparations. A size profile of a typical phospholipid preparation, where the soybean lecithin is suspended in saline and passed three times through a Manton-Gaulin homogenizer, then filtered through a 0.2 um filter was obtained. The average mean size of the liposomes was around 100nm in diameter, suggesting that the majority of liposomes resulting from this process are small unilameller vesicles (SUV's). The following experiment indicates that the size of the soybean lecithin liposomes may play an important role in the efficacy of the phospholipid, i.e., the ability of the SL to cause an increase in FVIII:c expression.

A soybean lecithin preparation was constituted in saline but not homogenized. The preparation contained significantly less SUV's than a normal (i.e., with homogenization) preparation (only 44% of the liposomes were below 100nms vs 74% in a normal prep). It also contained a significant population of multilamellar vesicles (MV's) which were around 500 nm's in diameter (30-40% of the total liposomes were MV's) which are present in only small quantities (usually < 5%) in normal preparations. The efficacy of this sample was reduced to about 60% of a normal sample indicating that the size of the liposomes may play an important role in the efficacy of the phospholipid.

Example IV

Porcine VWF can act to elicit Factor VIII:c activity from CHO cell propagated in the absence of serum.

Lig 1 (20uM MTX) cells were rinsed and fed defined media (alpha media containing insulin, transferrin, selenium, hydrocortisone, and putrescine, glutamine, and penicillin and streptomycin) added back with increasing concentrations of bovine serum albumin or with similar concentrations of ovalbumin Table II. Both proteins can act to elicit Factor VIII:c expression. However, when partially purified VWF is

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added back to media containing 5 g/l bovine serum albumin,
the Factor VIII:c activity increased four-fold to even
greater than the levels obtained upon propagation of the
cells in 10% fetal bovine serum. This dramatically demon-
strates the ability of VWF to elicit Factor VIII:c activity
in the absence of serum. This result has been duplicated
with different preparations of porcine VWF and also with
purified human VWF.

In order to demonstrate that the ability to elicit
factor VIII:c was due to VWF, the following experiment was
performed. Cells which express Factor VIII:c were incubated
in the presence of media containing serum derived from
human VWF deficient plasma. Factor VIII:c activity in the
CHO Lig 1 cells incubated in VWF deficient serum was 25%
the level compared to normal human serum. When the porcine
VWF preparation was added back to the VWF deficient serum,
the Factor VIII activity increased to the 10% fetal bovine
serum value. The effect could be elicited with as little as
2.50 ug/ml of VWF. See Table IIA. In another experiment,
when the VWF concentration was decreased to 0.25 ug/ml, the
activity was only 50% that of the 10% fetal bovine serum
level.

The CHO cell line 1E6 which had been adapted over a
2-3 month period to grow in the absence of serum, in defined
medium, was used in the following experiment in order to
demonstrate that exogeneous VWF could increase the level of
rVIII expression in defined medium in the absence of even
residual amounts of bovine VWF. The data presented below
shows that supplementation of defined medium with
approximately 1 microgram/mL of porcine VWF allows expression
of rFVIII equivalent to the level obtained in 10% fetal
bovine serum supplemented medium. Since these cells had
been grown for more than 3 months in the absence of FBS no
trace bovine VWF was present. Thus the observed increase
in rFVIII:c levels was likely due to exogeneous VWF.

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Effect of VWF on rFVIII Expression in the Absence of Serum

MEDIA	rFVIII (mU/ml)
Defined Media (DM)	~ 40
DM + VWF	~ 190
Media containing FBS (10%)	~ 200

This data illustrates the increase in the rFVIII activity by exogeneous, partially purified VWF (porcine) in the absence of fetal bovine serum. CHO cells (1E6 in 0.1 micromolar MTX) were suspended at a concentration of 3 x 10E5 cells/mL in defined medium containing 5g/L of bovine serum albumin either in the absence or presence of partially purified porcine VWF (at approximately 1 microgram/mL) or medium containing 10% FBS for 24h at 37 C. At the conclusion of the incubation samples of cell-free conditioned medium were assayed for rFVIII:c activity by chromagenic assay.

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Table II: Factor VIII:c Expression in Defined Media with
VWF Added back to Lig 1 Cells

Defined Media +		Units/ml/day
Ovalbumin (g/l)	0	0.164
	0.5	0.189
	1.0	0.215
	2.0	0.280
	5.0	0.290
	20.0	0.380
	5.0	
with procine VWF at 2.5 ug/ml		1.200
Defined Media +		
Bovine Serum Albumin		
(g/l)	0	0.190
	0.5	0.320
	1.0	0.380
	2.0	0.375
	5.0	0.430
	20.0	0.490
	5.0	
with porcine VWF at 2.5 ug/ml		1.350
10% Fetal Bovine Serum		0.978, 1.075

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Table IIA: Effect of VWF on Factor VIII Production

MEDIA	Mu/ml/day
10% fetal bovine serum	1321
defined media with 5 g/l bovine serum albumin	342
10% normal human serum	937
10% VWF deficient human serum	246
10% VWF deficient human serum with porcine VWF added back at:	
2.5 ug/ml	1124
20 ug/ml	1397

In order to examine the effect of added VWF on the amount of Factor VIII:c in the conditioned media, cells were labeled with a 1 hr. pulse of 35 - S methionine and chased in either media containing 10% fetal bovine serum, 10% VWF deficient human serum, or 10% VWF deficient human serum with porcine VWF added back. Results demonstrated that upon addition of VWF to VWF deficient serum, more Factor VIII:c (both the heavy 200kDa and the light 76kDa chains) was present in the media. No change in the intracellular synthesis of Factor VIII:c was observed. VWF addition to 10% fetal bovine serum resulted in no change in the level of Factor VIII:c in the conditioned media. These experiments indicate the VWF is necessary for the secretion and/or stability of Factor VIII:c.

EXAMPLE V

Expression of Human VWF in COS Cells

The cloning of a partial segment of the human VWF cDNA has previously been reported (Ginsberg, et al. 1985, Science). Subsequent to that report, the full length VWF cDNA has been assembled and its sequence determined. The cloning, sequence and expression of VWF have been described in detail inInternational Application No. PCT/US86/00760,

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published on 23 October 1986. We have inserted the full
length cDNA clone into the expression vector pMT2 to
produce pMT2-VWF (ATCC No. 67122). pMT2-VWF contains the
adenovirus associated (VA) genes, SV40 origin of replication
including the transcriptional enhancer, the adenovirus
major late promoter including the adenovirus tripartite
leader and a 5' splice site, a 3' splice site derived from
an immunoglobulin gene, the VWF coding region, a non-coding
DHFR insert, the SV40 early polyadenylation site, and the
pBR322 sequences needed for propogation in E. coli. Details
of this vector, which is a derivative of pQ2, are provided
in Kaufman, Proc. Natl. Acad. Sci., USA 82:689-693 (1985).
pMT2-VWF DNA was then prepared for COS cell transfection by
conventional methods. Sixty hours after DEAE dextran mediated
transfection of COS cells, the cells were labelled with
35-S methionine and media and cell extracts were
immuneprecipitated with a rabbit anti-human polyclonal
antibody (Calbiochem) and precipitates analyzed by SDS
reducing gel electrophoresis. Results demonstrate a
significant amount of VWF is synthesized in the transfected
COS cells, the majority of its being secreted. In the
conditioned media there is an approximately 260kDa protein
and a 220kDa protein which resembles the completely processed
form of VWF. Approximately 50% of the VWF synthesized is
processed to the 200kDa form. When analyzed for multimer
formation by non-reducing gel electrophoresis, it was found
the VWF was associated into multimers, but not of extremely
high molecular weight like those seen in plasma. The
multimers ranged up to 1 million daltons by a rough estimate.
Analysis of the VWF antigen in the COS cell conditioned
media indicated the presence of human VWF at 0.35 ug/ml.
Other analyses have indicated that the VWF expressed in COS
cells specifically binds both platelets and collagen.

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EXAMPLE VI

Recombinant VWF can elicit the expression of human Factor
VIII:c.

The VWF expression plasmid pMT2-VWF was transfected
onto COS cells by DEAE dextran mediated transfection and 36
hours post-transfection, the media changed to serum free
(DMEM lacking serum). 72 hours later the COS cell conditioned
media was harvested and applied to the CHO Lig 1 cells (20
uM MTX resistant) which were previously rinsed with serum-free
media (at 10^6 cells/ml). Twenty-four hours later the media
was taken from the CHO cells and assayed for Factor VIII
activity. The results are shown below and compared to
Factor VIII:c activities from CHO cells propagated in 10%
fetal bovine serum and in serum-free media for 24 hours.

Media on CHO Lig 1 (20uM MTX)	mU/ml Factor VIII:c
Conditioned media from mock transfected COS cells	141
Conditioned media from VWF transfected COS cells*	423
10% Fetal Bovine Serum	950
Serum-free media	30

*The conditioned media in this experiment contained 0.3
ug/ml of human VWF.

Example VII: Introduction, Expression, and Amplification of
VWF Genes in CHO Cells which express Factor VIII:c:

For expression of VWF in Chinese hamster ovary (CHO)
cells, a second expression vector, pMT2ADA-VWF (ATCC #67172),
was used with a protocol of selection for cells over-
expressing the enzyme adenosine deaminase to amplify the
plasmid sequences (Kaufman et al., 1986, Proc. Natl. Acad.

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Sci. 83:3136; U.S. Serial No. 619,801). A factor VIII:c expressing cell line which was cloned from ligl 2-a (from example I) in 1 mM MTX and designated 10Al, was used as recipient for transfer of pMT2ADA-VWF. pMT2ADA-VWF was introduced into 10Al cells by protoplast fusion as described (Sandri-Goldin et al., 1981, Mol. Cell. Biol. 1:743). E. coli DH5 cells harboring pMT2ADA-VWF (DH5 was used to minimize homologous recombination and deletion of the VWF sequences) were grown in 50 ml of L-broth containing 50 ug/ml ampicillin to an A_{600} of 0.6. Chloramphenicol was added to 250 ug/ml and the culture incubated at 37 C for an additional 16 hrs, in order to amplify the plasmid copy number. A suspension of protoplasts was prepared as described (Sandri-Goldin et al., 1981), added to 10Al cells at a ratio of approximately 1-2 X 10^4 protoplasts/cell, and centrifuged onto the cells at 2000 rpm for 8 minutes in an IEC model K centrifuge. After centrifugation, the supernatant was removed by aspiration and 2 ml of polyethylene glycol solution (50g of PEG 1450, Baker Chem. Co., in 50 ml of Dulbecco's modified medium) was added to each plate. Cells were centrifuged again at 2000 rpm for 90 seconds, the polyethylene glycol solution removed, and the plates rinsed 3 times in alpha medium containing 10% (v/v) fetal calf serum. Cells were then plated into tissue culture dishes in medium containing 100 ug/ml kanamycin, 10 ug/ml each of penicillin and streptomycin, and 20 uM MTX. Two days later the cells were trpysinized and subcultured 1:15 into ADA selective media with 10% dialyzed fetal calf serum, .1um deoxycoformycin, 10 ug/ml of penicillin and streptomycin, and in the presence and absence of 20 uM MTX. The ADA selective media (AAU) contained 1.1 mM adenosine, 10 ug/ml alanosine and 1mM uridine. Subsequently it was shown that removal of the MTX selection at this stage resulted in a decrease in the factor VIII:c expression. Subsequently, the MTX has been left in the ADA selective media.

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It was possible to amplify the VWF gene by selection for growth in increasing concentrations of 2'-deoxycoformycin (dCF) in the presence of cytotoxic concentrations of adenosine. A pool of transformants (6 colonies) was prepared from 10A1 cells 6 colonies in pool selected for ADA in the presence of 20 uM MTX. The ADA selection mean was changed by sequentially increasing the concentration of 2'deoxycoformycin (steps of 0.1 uM, 0.5 uM, 1.0 uM and 2.0 uM) in the presence of 20 uM MTX. At each step, the production of VWF and of factor VIII:c was measured after 24 hours in the presence of 10% fetal calf serum (FCS) or in defined media. The results are summarized below:

Coxpression of VWF and FVIII:c in CHO cell lines

Cell line	Selection		VWF Antigen		Factor VIII:c
	dCF uM	MTX uM	ug/ml	pg/cell	uUnits/cell
10A1 (no VWF)					.38* 0.93**
10A13 a pool	0.1	20	0.07	0.1	
	0.5	20	0.8	1.14	0.63* 0.89**
	1.0	20	24	30	0.63* 1.1**
	2.0	1000	7.4	24	1.4* 1.5*

*=in defined media; **= in media containing 10% Fetal calf serum; VWF antigen was determined by an ELISA assay using affinity-purified rabbit-anti-VWF antiserum (Calbiochem-Behring, 782301), purified VWF antigen from normal human plasma pools to serve as standards and controls, and IgG isolated from Calbiochem-Behring, 782301, and conjugated with alkaline phosphatase. Factor VIII:c activity was determined by the chromogenic assay described in Example I.

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These results demonstrate that VWF expression increased with increasing ADA selection. In addition, expression of factor VIII:c was not dependent on the presence of serum, as observed by line 10A13-a in 2 uM dCF and 1000 uM MTX which expresses 1.4 uUnits/cell/day of factor VIII:c in defined media.

Example VIII : Fusion of CHO cells expressing Factor VIII:c and CHO cells expressing VWF:

The VWF gene has been introduced into CHO DHFR deficient cells (DUKX-B11, Chasin and Urlaub, 1980, Proc. Natl. Acad. Sci. 77:44216). Two approaches have been taken in order to obtain cells that express either MTX resistance or dCF resistance associated with VWF expression. Then either cell line can be subsequently used to fuse to other cells that express factor VIII:c with the ability to select for either MTX or dCF resistance.

MTX Amplification in CHO DHFR deficient Cells

Plasmid pMT2VWF and pAdD26SVpa(3) were mixed 10:1 and transfected by CaPO$_4$ coprecipitation into CHO DUKX-B11 cells as described by Kaufman and Sharp (1982, J. Mol. Biol. 150:601). Cells were selected for the DHFR positive phenotype by growth in the absence of nucleosides and colonies pooled and selected for increasing MTX resistance. The results indicated that VWF expression increased with increasing MTX resistance and are depicted in the Table below:

Selection	ng/ml VWF
0.02 uM MTX	– – – –
0.2 uM MTX	56
1.0 uM MTX	91
5.0 uM MTX	278

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dCF Selection for VWF in CHO DHFR Deficient Cells

The plasmid pMT2ADA-VWF was introduced into CHO DUKX-B11 cells as described in Example VIII and cells selected for growth in ADA selective alpha media with 4 uM xyl-A, 0.03 uM dCF, 10 ug/ml hypoxanthine, 10 ug/ml thymidine, and 10 ug/ml of penicillin and streptomycin. One clone PM5F was derived which expressed 3-5 pg of VWF/cell/day. This clone was subsequently used for fusion to factor VIII:c cell lines and as a recipient for the introduction of factor VIII:c genes.

Fusion of Factor VIII:c and VWF Expressing Cell Lines

The factor VIII:c expression plasmid pLA2 has been described (Toole et al., 1986, Proc. natl. Acad.; International Application No. PCT/US86/00774). This plasmid has been introduced into DUKX-B11 CHO cells by protoplast fusion with selection for DHFR from the 3' region of the factor VIII-DHFR transcript (See PCT/US86/00774). A cell line was derived by selection for MTX resistance to 1.0 uM MTX and has been named LA3-5. This cell line expresses a deleted form of Factor VIII:c at 3-5 uUnits/cell/day (in 10% fetal calf serum). This modified factor VIII:c also binds to and requires VWF for efficient synthesis. LA3-5 was fused to PM5F and hybrids were selected that expressed both the MTX resistance and dCF resistance phenotypes.

For fusion, PM5F was treated with diethylepyrocarbonate (0.03% for 30 minutes on ice) in order to kill the PM5F. These cells were then fused by polyethylene glycol-induced cell fusion to LA3-5: DEPC treated pMSF cells were centrifuged onto LA 3-5 (1.5 X 10^6 cells) at 2000 rpm for 8 minutes in an IEC model K centrifuge. After centrifugation, supernatant

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was removed and 2 ml of 50% PEG solution was added. PEG
was left on for 45 seconds and cells were washed thoroughly
with serum free medium. Cells were left plated with medium
containing serum for 48 hrs. and were then subcultured into
selective medium containing 4uM xyl-A, 0.03 uMdCF, in the
presence of 10 ug/ml of each of the following: thymidine,
hypoxanthinine, streptomycin, and penicillin. However, it
was not necessary to include the thymidine and hypoxanthine.
A pool of hybrids was obtained which expressed 0.73
pg/cell/day of VWF and 0.2 - 2.0 units/ml/day of factor
VIII:c. The pool was subsequently grown in the absence of
thymidine and hypoxanthine in the presence of 0.5 uM MTX.
These cells were cloned in alpha media with 4 uM xyl-A,
0.03 uM dCF, and 0.5 uM MTX to obtain the following clones:

Factor VIII:c and VWF Coexpression in CHO Cells

Clone	VWF Expression (pg/cell)	Factor VIII:c Expression (uUnits/Cell-media)
E6	16	2.8 - defined 3.8 -10% FCS
B9	20	4.5 - defined 5.1 - 10% FCS
H6	34	7.7 - defined 8.7 - 10% FCS
B1	8.	10.5 defined 11.8 10% FCS

These results demonstrate the ability of the VWF and Factor
VIII:c co-expressing cell lines to produce high levels of
factor VIII:c in defined media.

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Introduction of Factor VIII:c Gen s into Cells Expressing VWF

A factor VIII:c deletion mutant of 907 amino acids has been constructed by heteroduplex mutagenesis (PCT/US86/00774) which directly fuses the 90 kDa cleavage site (at residue 740) to the 76 kDa cleavage site (at 1647). The resultant plasmid p90-76R has the factor VIII:c coding region 5' on the polycistronic transcription unit in pMT2. Protoplasts of E. coli HB101 harboring this plasmid were prepared and fused to the VWF expressing cell line PM5F as described in Example VIII. 48 hrs after recovery from protoplast fusion, the cells were subcultured into DHFR selection media (alpha media lacking nucleosides with 10% dialyzed fetal calf serum, 4uM xyl-A, and 0.03 uM dCF. After two weeks, transformants were isolated and assayed for Factor VIII:c expression. Approximately 20% of the transformants which had arisen expressed both VWF and Factor VIII:c. Results for one transformant are indicated below:

Cell Line	Factor VIII:c Activity	
F1	1.5 uUnits/cell	1375 mUnits/ml def. media
	0.95 uUnits/cell	1330 mUnits/ml 10% FCS

(with VWF= 1.69 ug/ml, 1.85 pg/cell)

These results demonstrate the ability to select for the DHFR phenotype in the PM5F cell line and to coexpress factor VIII:c and the VWF in order to alleviate the serum dependence for factor VIII:c expression.

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What is claimed is:

1.   In a method for the production of a Factor VIII:c-type protein which comprises culturing mammalian cells capable of expressing the Factor VIII:c-type protein, the improvement comprising culturing the cells in a cell culture medium which contains less than about 10% mammalian serum (by volume) and contains a composition selected from the group consisting of:

(a) mammalian von Willebrand Factor (VWF);
(b) a phospholipid or phosopholipid mixture; and
(c) a mixture of (a) and (b).

2.   A method of claim 1, wherein the composition is VWF and the medium contains not more than about 1% mammalian serum by volume.

3.   A method of claim 2, wherein the medium is essentially serum free.

4.   A method of claim 1 wherein the VWF comprises VWF derived from mammalian serum.

5.   A method of claim 1 wherein the VWF comprises recombinant mammalian VWF.

6.   A method of claim 4 wherein the medium contains the recombinant VWF by virture of being conditioned by genetically engineered cells which produce the VWF.

7.   A method of claim 6 wherein the genetically engineered cells which produce the VWF co-express the Factor VIII:c.

8.   A method of claim 6 wherein the recombinant VWF is produced separately from the Factor VIII:c and is added to the medium in which the Factor VIII:c-producing cells are

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cultured.

9.   A method of claim 1 wherein the phospholipid or phospholipid mixture is selected from the group consisting of Cephalin, phosphatidyl serine; phosphatidyl choline, phosphatidyl inositol, phosphatidyl ethanolamine, soybean lecithin or a mixture thereof.

10.   A method of claim 1, wherein the phospholipid is soybean lecithin.

11.   A method of claim 10, wherein the SL is present in an amount between about 1 and 1000 ug SL/ml of medium.

12.   A method of claim 11, wherein the SL is present in an amount between about 100 and 300 ug SL/ml of medium

13.   A method of claim 10, wherein the medium contains less than about 5% serum by volume.

14.   A method of claim 13 wherein the medium contains no more than about 1% serum by volume.

15.   A method of claim 1, wherein the phospholipid or phospholipid mixture is in the form of dried milk in an amount from about 0.01 to about 10% by weight based on the volume of medium.

16.   A method of claim 15, wherein the amount of dried milk is from about 1 to about 3% by weight based on teh volume of medium.

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S. Cl. 435/70

Int. Cl. (4) C 12 P 21/02

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴	
Classification System	Classification Symbols
U.S	435/68, 70, 172.3, 240 935/70

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵
CHEMICAL ABSTRACTS, BIOSIS; FACTOR VIII and PHOSPHOLIPID or WILLEBRAND.

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	WO 8,501,961, (GENETICS INSTITUTE), 09 May 1985, See Abstract.	1-16
Y	NATURE 312, 22 November 1984, WOOD ET AL "Expression of Active Human Factor VIII from Recombinant DNA Clones", pages 330-337.	1-16
Y	JOURNAL OF CLINICAL INVESTIGATION 60 August 1977, WEISS ET AL, "Stabilization of Factor VIII in Plasma by the von Willebrand Factor", pages 390-404.	1-8
Y	BIOCHEMICAL JOURNAL 200, 1981, ANDERSON ET AL, "Interaction of Factor VIII - von Willebrand Factor with Phospholipid Vesicles", pages 161-167.	1-5 and 9-14

* Special categories of cited documents: ¹⁵

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the International filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ²
11 March 1987	2 4 MAR 1987
International Searching Authority ¹	Signature of Authorized Officer
ISA/US	John E. Tarcza

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET		
Y	CELL 41, May 1985, LYNCH ET AL, "Molecular Cloning of cDNA for Human von Willebrand Factor: Authentication by a New Method", pages 49-56.	6-8
Y	JP,A 0013389, (KYOWA HAKKO KOGYO), 25 January 1983, See Abstract.	1-5 and 9-14
Y	JP,A 0023784, (AJINOMOTO), 12 February 1983, See Abstract.	1-5 and 9-14

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 10

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers ...., because they relate to subject matter 12 not required to be searched by this Authority, namely:

2.☐ Claim numbers..........., because they relate to parts of the International application that do not comply with the prescribed require-ments to such an extent that no meaningful International search can be carried out 13, specifically:

VI.☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 11

This International Searching Authority found multiple inventions in this International application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2) (May 1986)

III. DOCUMENTS C NSIDERED TO BE RELEVANT   (C NTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	EP 0112174, (IMMUNEX), 27 June 1984 See Abstract.	1-5 and 9-14
Y	JA 0166079, (MORINAGA), 19 September 1984, See Abstract.	15 and 16
	JA 0173078, (MORINAGA) 29 September 1984, See Abstract.	15 and 16